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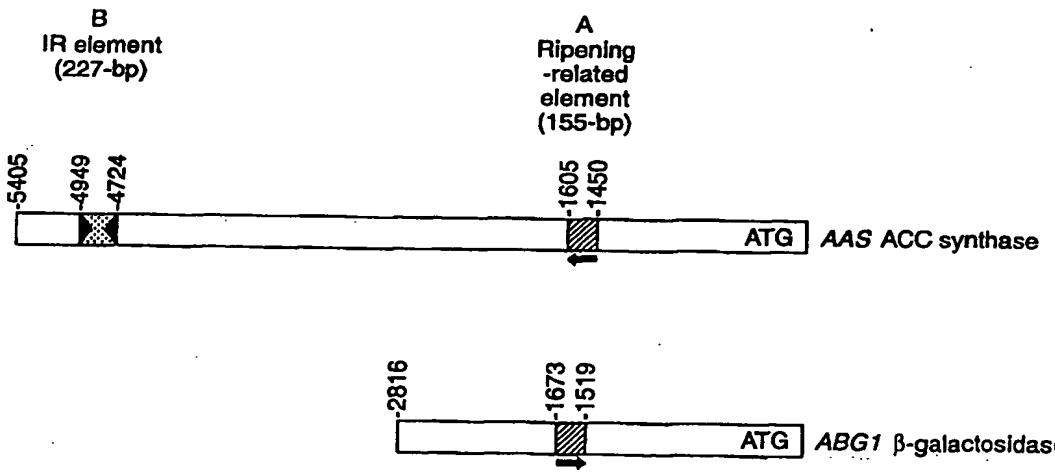

(57) Abstract

A recombinant polynucleotide comprising a promoter sequence being: (a) an inducible promoter obtainable from apple, or (b) a functional portion thereof, or (c) a functional derivative or homolog promoter being at least 70 % homologous to either. The promoter sequence is preferably activated in response to which agents are specific to ripening fruit and is most preferably the apple β–Galactosidase (ABG1) promoter, or the 1–AminoCyclopropane–1–Carboxylate synthase (ACC Synthase) promoter. Vectors form a further part of the invention. Also provided are host plant cells, plus methods of producing transgenic plants and fruit which incorporate antisense RNA capable of down–regulating genes involved in ripening or peptides or proteins improving fungal, insect, bacterial, viral, herbicidal, nematode, or arachnid resistance. Such transgenic plants and fruit have storage and pest–resistance properties superior to non–transgenic varieties.

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INDUCIBILE PLANT PROMOTERS

TECHNICAL FIELD

The present invention relates to polynucleotides which may be useful in recombinant plant DNA technology or analysis, in particular to tissue- or ripening-specific promoter DNA, and products and methods employing such DNA.

BACKGROUND ART

It is desirable to be able to specifically express (or inhibit the expression of) genes in plants, for instance in particular tissues, or at a particular developmental stage. This may allow particular biosynthetic enzymes to be produced only in the fruit of a plant, and not in other tissues wherein it may have undesirable effects. Likewise it may be desirable to have particular protective proteins (e.g. anti-fungal, pesticidal) expressed only during a particular vulnerable developmental stage e.g. early or late ripening.

This type of specific expression can be achieved by using inducible promoters which are 'switched on' in the presence of environmental signals present only in restricted tissues of the plant, or only at particular times. Such promoters have already been made available for tomatoes. Thus WO93/07257 (SPI Inc.) relates, *inter alia*, to gene-fusions capable of conferring tissue-specific or developmentally regulated gene constructs. These constructs apparently allow particular genes to be expressed during the formation and ripening of fruit. The coding region of clone λUC82-3.3 in WO93/07257, which was derived from tomato, has homology to a bacterial histidine decarboxylase (HDC). Similarly WO94/13797 (CSIRO) relates, *inter alia*, to inducible soft-fruit promoter DNA derived from alcohol

dehydrogenase (ADH) in tomatoes. ADH apparently has a role in ripening in that it metabolises alcohols and aldehydes involved in flavour. The ADH promoter is apparently sensitive to and therefore inducible by high levels of O_2.

It is clear from the foregoing that the disclosure of novel inducible promoters, particularly those active in plants other than tomato plants, would provide a useful contribution to the art.

The applicants have now isolated inducible promoters from apple, elements of which show useful properties and which may be useful in particular in the isolation of other ripening specific promoters or transcription factors, or in the genome mapping studies.

DISCLOSURE OF THE INVENTION

In a first aspect of the present invention there is disclosed a recombinant polynucleotide comprising a promoter sequence being: (a) an inducible promoter obtainable from apple, or (b) a functional portion therof, or (c) a functional derivative or homolog promoter being at least 70% homologous to either.

As used herein, "promoter" refers to a non-coding region of DNA involved in binding of RNA polymerase and other factors that initiate or modulate transcription whereby an RNA transcript is produced. Promoters, depending upon the nature of the regulation, may be constitutive or inducible. A constitutive promoter is always turned on. An inducible promoter requires specific signals in order for it to be turned on or off. These may be particular signals for example chemical signals, which are applied to a cell under certain conditions or as a result of a deliberate application. In the context of the present

application, the term "inducible" is intended to include particularly promoters which are tissue-specific in that they are effective only in certain plant tissues either with or without externally applied inducing agents, or ripening specific promoters which switched on within some or all plant cells as a result of ripening, for example in response to ethylene produced during the ripening process.

Examples of promoters of the invention include a ABG1 β-galactosidase promoter whose sequence is included within the sequence shown in Figure 3 hereinafter (SEQ ID NO 1); and the ACC synthase promoter whose sequence is comprised within the sequence shown in Figure 5 (SEQ ID NO 2) hereinafter.

Thus, the invention provides a promoter comprising at least a functional portion of the Sequence shown in Figure 3 or Figure 5.

As well as authentic promoters obtainable from apple, the invention also embraces functional portions thereof.

The term "functional" is used herein to describe moieties which have the activity of a promoter as defined above, when present in apple cells.

Also embraced by present invention are functional derivative promoters being at least 70% homologous to the above.

By "derivative" is meant a sequence may be obtained by introducing changes into the full-length or part length sequence, for example substitutions, insertions, and/or deletions. This may be achieved by any appropriate technique, including restriction of the sequence with an endonuclease followed by the insertion of a selected base sequence (using

linkers if required) and ligation. Also possible is PCR-mediated mutagenesis using mutant primers. Such changes may be introduced e.g. to remove or incorporate restriction sites into the sequence.

Also embraced by the present invention are functional "homologs" of authentic promoters obtained from apple which hybridise thereto and are at least 70% homologous to either the full-length or part length sequences and in particular to SEQ ID NOS 1 and 2 identified herein.

Such homologs may conveniently be identified and isolated by those skilled in the art from a test sample as follows:

The test sample is contacted with the apple promoter under suitable hybridisation conditions, and any test DNA (e.g. an apple genomic library) which hybridises thereto is identified.

Such screening is initially carried out under low-stringency conditions, which comprise a temperature of about 37°C or less, a formamide concentration of less than about 50%, and a moderate to low salt (e.g. Standard Saline Citrate ('SSC') = 0.15 M sodium chloride; 0.15 M sodium citrate; pH 7) concentration.

Alternatively, a temperature of about 50°C or less and a high salt (e.g. 'SSPE'= 0.180 mM sodium chloride; 9 mM disodium hydrogen phosphate; 9 mM sodium dihydrogen phosphate; 1 mM sodium EDTA; pH 7.4). Preferably the screening is carried out at about 37°C, a formamide concentration of about 20%, and a salt concentration of about 5 X SSC, or a temperature of about 50°C and a salt concentration of about 2 X SSPE. These conditions will allow the identification of sequences which have a substantial degree of similarity with the probe sequence, without requiring perfect homology for the identification of a

stable hybrid. The phrase 'substantial similarity' refers to sequences which share at least 50% overall sequence identity. Preferably, hybridisation conditions will be selected which allow the identification of sequences having at least 70% sequence identity with the probe, while discriminating against sequences which have a lower level of sequence identity with respect to the probe.

After low stringency hybridisation has been used to identify one or more homologs having a substantial degree of similarity with the probe sequence, this subset is then subjected to high stringency hybridisation, so as to identify those clones having a particularly high level of homology with respect to the probe sequences. High stringency conditions comprise a temperature of about 42°C or less, a formamide concentration of less than about 20%, and a low salt (SSC) concentration. Alternatively they may comprise a temperature of about 65°C or less, and a low salt (SSPE) concentration. Preferred conditions for such screening comprise a temperature of about 42°C, a formamide concentration of about 20%, and a salt concentration of about 2 X SSC, or a temperature of about 65°C, and a salt concentration of about 0.2 SSPE.

Thus, according to the present invention the derivative sequence or homolog is at least 70% identical to the sequence of the full or part- length promoters. Typically there is 80% or more, 90% or more 95% or more or 98% or more identity between the derivative or homolog and the authentic sequences. There may be up to five, for example up to ten or up to twenty nucleotide deletions, insertions and/or substitutions made to the full-length or part length sequences.

Whether a part-length or modified or homologous sequence is capable of acting as a promoter (is "functional") may be readily ascertained in the light of the present disclosure by those skilled in the art. Briefly, the candidate sequence is provided in a vector upstream of a protein coding sequence at a position in which it is believed to be operatively linked to that coding sequence. A suitable host cell, preferably an apple cell, is transformed with the resulting vector. The presence or absence of the protein coded by the sequence is determined.

Preferably the polynucleotide of the first aspect comprises a promoter sequence which is activated in response to tissue specific agents i.e. is turned on or off as a function of the tissue in which it is present. More preferably the agents are specific to fruit, and most preferably specific to ripening fruit (i.e. the promoter is a developmentally regulated promoter which is turned on or off as a function of development).

Two particular examples of promoter sequences of the invention are the Apple β-Galactosidase (ABG1) promoter, or the 1-AminoCyclopropane-1-Carboxylate synthase (ACC Synthase) promoter. Isolated, non-recombinant, polynucleotides encoding these promoters, or functional portions or dervatives or homologs thereof form a further part of the present invention. The sequences of these promoters are included within the sequences given hereinafter in Figures 3 and 5 respectively and recombinantly produced or synthetic promoters comprising or derived from these sequences also fall within the ambit of the invention.

Computer-assisted examination of the DNA sequences of the *ABG1* (2879-bp) and the *AAS* ACC synthase (5391-bp) promoter containing fragments of Figures 3 and 5 has shown the presence of some

interesting sequence motifs as illustrated in Figures 7 and 8 below (SEQ ID NOS 3 and 4 and 5 and 6 respectively). These motifs form preferred examples of portions of the *ABG1* and *AAS* ACC synthase promoters.

A: At approximately the same location (1.5-1.6-kbp) upstream from the start codon of these two ripening-related genes there is a highly conserved sequence of 155-bp. The orientation of the sequence is opposite in the two promoters (SEQ ID NOS 3 and 4). These two sequences are 90% similar and contain an unusual repeat element (GAAAAATCACATTTTTACACTAAAAAG -SEQ ID NO 7) or a derivative thereof, which has dyad symmetry about the central T residue. This unit is found in the ACC synthase promoter sequence (Figure 8) and is varied only by two conservative (T→C) substitutions in the ABG1 sequence. This is believed to be the binding site for a dimeric transcription factor, and considering the extent of conservation of the DNA sequence encompassing this motif, it may be involved in the regulation of transcription during fruit ripening.

This 155 bp DNA sequence could be used as a probe fragment to isolate other ripening-specific promoters by library screening, for example as described above.

Furthermore, as it is likely to be important in ripening-specific gene expression, the sequence could be used as a component of a minimal promoter. Removal of extraneous non-functional sequences is desirable to satisfy regulatory considerations and would reduce the size of promoters considerably, making them more versatile.

Thus in a preferred embodiment, the invention provides a inducible promoter which comprises SEQ ID NO 3 or SEQ ID NO 4 or a functional portion thereof, or a functional derivative or homolog promoter being at least 70% homologous to either. Preferably, the promoter will comprise SEQ ID NO 3 of SEQ ID NO 4.

These sequences could be used in strategies to isolate transcription factors involved in ripening-specific gene expression. They could be coupled to magnetic beads to affinity purify proteinaceaous factors from extracts of fruit cell nuclei or could be radiolabelled and used to screen a fruit cDNA expression library. Such methods form a further aspect of the invention.

B: Another notable sequence occurs approximately 4.7-kbp upstream of the start codon in the ACC synthase promoter (Figure 7). This sequence (SEQ ID NO 5 in Figure 8) of 227-bp has Inverted repeat (IR) elements at its termini. The only significant similarity identified is with a sequence (217-bp) seen in the promoter of an apple kn1-like knotted gene homologue [Watillon, B. (1996), *M.domestica* partial gene for kn1-like protein. GB accession Z71981- SEQ ID NO 6]. The homology is 61% overall, but considerably higher at the termini.

A PCR fragment encompassing the apple kn1-like IR element has been used to probe a Southern blot of genomic DNA. This showed that there are multiple copies of the element in the apple genome and appears to confirm that the sequences represent transposable inverted repeat elements. The identification of such elements has never before been reported in apple.

These elements share some features with the *Stowaway* class of IR elements [Bureau, T.E. and Wessler, S.R. (1994) *Stowaway*: A new family of inverted repeat elements associated with the genes of both monocotyledonous and dicotyledonous plants. The Plant Cell 6: 907-916]. *Stowaway* and similar plant IR elements may represent transposable elements, their remnants after transposition or solo terminal repeats from a larger element.

The apple IR element identified is similar in size to *Stowaway* elements found in dicotyledonous plants (248bp +/- 24bp) and is also AT rich. It differs in the target site for insertion (TA in *Stowaway*) and the nature of the conserved terminal repeat region. It therefore represents a new class of element which has not been reported previously.

The inverted repeat element may be of use in genome mapping in rosaceous species. Depending on how widespread it is and in what copy numbers it is found, it may be used in a similar way to microsatellites. Such methods form yet a further aspect of the invention.

In a further aspect of the invention there is provided a replication vector comprising a polynucleotide as described above and further comprising a replication element which permits replication of the vector in a suitable host cell.

"Vector" is defined to include, *inter alia*, any plasmid DNA, lysogenic phage DNA and/or transposon DNA, in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable and which can transform prokaryotic or eukaryotic host either by integration into the cellular genome or exist extrachromosomally (e.g. autonomous replicating

plasmid with an origin of replication). Introduced by any method e.g. conjugation, mobilisation, transformation, transfection, transduction or electroporation. The term explicitly includes shuttle vectors by which is meant a DNA vehicle capable, naturally or by design, of replication in both bacterial and plant cells.

In yet a further aspect of the invention there is provided an expression vector comprising a polynucleotide as described above. Preferably the vector further comprises a heterologous gene operatively linked to said promoter sequence.

As used herein, the terms "operatively linked" denotes the linkage of a promoter or a non-coding gene regulatory sequence to an RNA-encoding DNA sequence, and especially to the ability of the regulatory sequence or promoter to induce production of RNA transcripts corresponding to the DNA-encoding sequence when the promoter or regulatory sequence is recognised by a suitable polymerase.

Preferably the heterologous gene encodes any of: (a) antisense RNA capable of down-regulating genes involved in ripening; (b) a peptide or protein improving fungal, insect, bacterial, viral, herbicidal, nematode, or arachnid resistance; (c) a detectable or selectable marker protein. Examples of some of such heterologous genes are known to those skilled in the art (see e.g. WO93/07257, WO94/13797). Ripening specific genes include those involved in ethylene biosynthesis or cell wall degradation. Proteins involved in fungal degradation include β-1,3-glucanases and chitinases. Marker proteins include β-glucuronidase (GUS).

Preferably the vector comprises elements derived from disarmed strains of *Agrobacterium tumefaciens*, such as are known to those in the art.

The invention further provides a host cell containing a vector as described claimed above, or transformed with such a vector. Typically the host cell will constitute all or part of a plant protoplast, plant callus, plant tissue, developing plantlet, or immature whole plant. The plants/cells may be apple or other fruit in which the promoters are functional (e.g. tomato, melon, strawberry).

In addition, the invention provides a method of producing a transgenic plant comprising regenerating a mature plant from the transformed host cell described above.

As used herein, "transgenic" plants refer to plants or plant compositions in which heterologous or foreign DNA is expressed or in which the expression of a gene naturally present in the plant has been altered. Such heterologous DNA will be in operative linkage with plant regulatory signals and sequences. The DNA may be integrated into a chromosome or integrated into an episomal element, such as the chloroplast, or may remain as an episomal element. In creating transgenic plants or plant compositions, any method for introduction of such DNA known to those of skill in the art may be employed. A transgenic plant comprising such a host cell, either produced as described above or by further propagation of transgenic plants forms a sixth aspect of the invention.

A further aspect of the invention provides a method of producing apples having a modified phenotype, said method comprising cultivating a transgenic apple plant described above and

harvesting the fruit of the plant. The fruit itself forms yet a further aspect of the invention.

The invention will now be further described with reference to the following non-limiting examples. Further embodiments falling within the scope of the invention will occur to those skilled in the art in the light of these.

FIGURES

Figure 1 shows a sequence comparison between the ABG cDNA disclosed by Ross, G.S., Wegrzyn, T-, MacRac, E-A- & Redgwell, R.J. (1994) " Apple β-galactosidase: Activity against cell wall polysaccharides and characterisation of a related cDNA clone" Plant Physiology 106: 521-528 and the *Eco*RI (Figure 1(1)) and *Pst*I fragments (Figure 1(2)) of the genomic clone which contained the ABG1 promoter. In this figure, the upper sequences (SEQ ID NO 8 and 9) are those of the Ross et al. cDNA. The lower sequences (SEQ ID NO 10 and No 11 respectively) in italics are those of the genomic clone. Hyphens mark gaps introduced for alignment or introns. Differences (or Ns) in the genomic sequences are double underlined and amino acid residues at intron boundaries are numbered. Differences in amino acid sequence are indicated beneath sequence in bold, italics and underlined.

Figure 2 shows a sequence comparison between the ACC synthase cDNA disclosed by Lay-Yee, M & Knighton, M L 1995) "A full length cDNA encoding 1-aminocyclopropane-1-carboxylate synthase from apple" Plant Physiology 107:1017-1018 (SEQ ID NO 12) and part of the genomic clone which contained the ACC synthase promoter (SEQ ID NO 13).

Figure 3 shows a sequence of a region of the ABG1 β-galactosidase gene which is upstream of the coding region incorporating a promoter sequence (SEQ ID NO 1).

Figure 4 shows the upstream sequence of the the ABG1 β-galactosidase gene including the promoter sequence but terminating at the start codon ATG (SEQ ID NO 14) together with its complementary strand (SEQ ID NO 15) sequence, annotated with restriction sites.

Figure 5 shows a sequence of a region of the ACC synthase gene which is upstream of the coding region incorporating a promoter sequence (SEQ ID NO 2).

Figure 6 shows the upstream sequence of the ACC synthase gene including the authentic promoter sequence including the start codon (ATG) from which the coding sequence has been removed (SEQ ID NO 16) and its complementary strand (SEQ ID NO 17) annotated with restriction sites.

Figure 7 shows sequence features of promoters of the invention.

Figure 8 shows the alignment of the features illustrated in Figure 7 in the ABG1 and AAS promoters.

EXAMPLES

SOURCE OF MATERIALS USED IN THE ISOLATION OF FRUIT-RIPENING-SPECIFIC PROMOTERS

ABG1 β-galactosidase cDNA

Plant: *Malus domestica* [Borkh] cv Granny Smith
Tissue: Mature unripe fruit cortex
Construct: pABG1

Vector: pBluescript II SK
cDNA Insert: 2637bp
Accession: L29451
Reference: Ross et al (1994) [supra].
Location: The Horticulture and Food Research Institute of New Zealand Ltd., Mt- Albert Research Centre, Private Bag 92 169, Auckland, New Zealand.

ACC Synthase CDNA

Plant: *Malus sylvestris* Mill., cv. Golden Delicious
Tissue: ripe apple fruit mesocarp
Construct: pAAS2
Vector: pCGN1703
CDNA Insert: 1636bp
Accession: U03294
Reference: Dong, J.-G., Kim, W.-T., Yip, W.-K., Thompson, G.A., Li, L., Bennett, A-B. & Yang, S.-F.(1991) " Cloning of a CDNA encoding 1-aminocyclopropane-1-carboxylate synthase and expression
of its MRNA in ripening apple fruit" Planta 185: 38-45
Location: Mann Laboratory, Department of Vegetable Crops, University of California/Davis, CA 95616, USA. Note this is practically identical to the full length Lay-Yee and Knighton clone (1995) [supra] used in the sequence comparison.

Apple genomic library

Plant: Malus domestica [L] Borkh cv Mcintosh 'Wijcik'
Tissue: Nuclei isolated from *in vitro* propagated apple leaves
Vector: Lambda Gem 11 (Promega)
Construction: Partially *Sau*3A digested DNA ligated to *Xho*I half-site vector arms

Reference: Watillon, B., Kettmenn, R., Boxus, P. & Burny, A. (1992) "Cloning and characterization of an apple (Malus domestica [L.] Borkh) calmodulin gene" Plant Science 82:201-212 Location: Faculté des Sciences Agronomiques, Unité de Biologie Moléculaire et Physiologie Animale and Centre de Recherches Agronomiques, Station des Cultures fruitierès et maraîchères, 5030 Gembioux, Belgium.

EXAMPLE 1: PROCEDURE FOR THE ISOLATION OF FRUIT-RIPENING-SPECIFIC PROMOTERS FROM APPLES

Throughout the procedure for the isolation of fruit-ripening-specific promoters, standard protocols as described by Sambrook *et al* (1989) "Molecular cloning: a laboratory manual (2nd edition)" Cold Spring Harbor Laboratory Press, were used except where indicated. DNA probe fragments were prepared by restriction digestion of plasmids containing the ABG1 and AAS (encoding ACC Synthase) cDNAs with restriction endonucleases *Hind*II and *Eco*RI respectively. The ABG 1 (1182bp) and AAS (approx. 740bp) fragments were gel purified using the BIO 101 inc. geneclean 11 kit and labelled with digoxigenin by the random priming procedure supplied by the DIG kit manufacturers (Boehringer Mannheim UK Ltd.). The apple genomic library was plated and the plaques replicated on a nylon membrane by lifting. Hybridization was performed using HYBSOL buffer (Yang *et al*, (1993) Nucleic Acid Research 21:3337-3338) using conditions recommended in the DIG kit protocol. The hybridization temperature was 68°C and the post-hybridization wash conditions were stringent to ensure that only homologous DNA sequences were identified. Specific hybridization of the probes to lambda plaques containing homologous sequence was detected by chemiluminescence following the DIG kit protocol.

Single positive plaques hybridizing with the ABG1 and ACC synthase probes were identified and named λABG1 and λAAS respectively. These were purified to homogeneity by further rounds of plaque lifting and hybridization with the specific probes. The positive phages were then propagated and phage DNA was prepared from these amplified stocks using the lambda DNA purification kit from Promega Ltd. The purified DNA was digested using a panel of restriction endonucleases and the fragments resolved on an agarose get. The gel was Southern blotted onto Hybond-N Nylon (Amersham Ltd.) using standard protocols and the blot probed with the DIG-labelled DNA fragments to identify bands containing homologous sequence. Hybridization was again detected by chemiluminescence.

Repeat large-scale digests were performed with the selected endonucleases and positive DNA bands gel purified using the geneclean II kit. The isolated bands were then cloned into the plasmid vector pGEM-3Zf(+) (Promega Ltd.). Recombinant plasmid DNA was prepared from small cultures using a plasmid miniprep. kit from QIAGEN Ltd. This DNA was used as the template for cycle sequencing reactions using the PRISM dye-terminator cycle sequencing kit from Applied Biosystems Ltd. Separate sequencing reactions of each construct using T7 and -21 M13 forward primers were performed and analyzed by the DNA sequencing service at the University of Durham (UK) using an Applied Biosystems DNA sequencer.

The sequences of each fragment combined with restriction mapping data established the identity of the genes as ABG1 and AAS and allowed the location of the cloned DNA fragments to be established (Figure 1). To 'DNA walk' within the lambda clones to identify DNA fragments encompassing the promoter sequences, new probes were prepared from the cloned fragments. These were

used to re-probe the lambda DNA Southern blots to identify large fragments predicted to cover the promoter region of each gene. These fragments were again cloned into plasmid vector pGEM3Z(f)+ and characterized by restriction mapping and sequencing of the termini.

Figure 1 shows a sequence comparison between the ABG cDNA (SEQ ID NO 8 and 9) disclosed by Ross et al (1994) [supra] and the *Eco*RI and *Pst*I fragments of the genomic clone containing the ABG1 promoter (SEQ ID NO 10 and SEQ ID NO 11 respectively). Note that the sequence 5' of the start of the cDNA sequence represents part of the region containing the promoter.

Figure 2 shows a sequence comparison between the ACC synthase cDNA disclosed by Lay-Yee & Knighton 1995) Plant Physiology 107:1017-1018 (SEQ ID NO 12) and part of the genomic clone containing the ACC synthase promoter (SEQ ID NO 13). Note that the sequence 5' of the start of the cDNA sequence represents part of the region containing the promoter.

Deletion of small restriction fragments from these large fragments, followed by DNA sequencing allowed the determination of the sequences flanking the ATG start codon of each gene to be determined. This information was used to devise strategies to subclone the promoters to drive marker gene (*gusA* or *uidA*) expression in a plant transformation vector. The precise subcloning strategies are given below:

SUBCLONING STATEGIES

ABG1 β-galactosidase

a. λABG1 isolated by probing genomic library with ABG1 cDNA *Hind*III fragment.

b. *5.5kb Sph*I apple ABG1 genomic fragment isolated from λABG1

c. Ligated to *Sph*I digested pGEM3Z and obtain clone with *Eco*RI site of vector at the 3' end of the promoter ABG1 fragment = pGEM3ZABG1*Sph*(2)

d. Digested pGEM3ZABG1*Sph* with *Pst*I to excise 2.8kb fragment containing coding sequence, and ligated to recircularise = pGEM3ZABG1*SphΔPst*(1)

e. Digested pGEM3ZABG1*SphΔPst* with *Bsr*DI and blunt ends with T4 pol. Then digested with *Eco*RI and isolated 525bp *Bsr*DI(blunt)/*Eco*RI fragment.

f. Partially digest pGEM3ZABG1*SphΔPst* with *Eco*RI (2 sites present) and isolated linearised form.

g. Digested linearised form with *Sma*I to cleave downstream in the multiple cloning site and isolated band released by *Eco*RI cleavage within the ABG1 promoter (not the *Eco*RI site in the multiple cloning site).

h. Ligated pGEM3ZABG1*SphΔPst (Eco*Rlpartial/*Sma*I) with 525bp *Bsr*DI *blunt 1Eco*RI frag pABG1P (4).

i. Digested pABG1P with *Sac*I *and Sph*I to release 2.7kb ABG1 promoter fragment.

j. Treated with T4 pol to blunt *Sac*I and *Sph*I ends of promoter fragment

k. Ligated to *Sma*I digested pSCV1.6 and isolate recombinant carrying promoter fragment in correct orientation = **pSCV1.6ABG1P** (5)

ACC Synthase

a. λAAS isolated by probing genomic library with AAS cDNA *Eco*RI fragment. A 7kb *Sac*I apple AAS genomic fragment is isolated from λAAS

b. Ligated to *Sac*I digested pGEM3Z and obtained clone with *Eco*RI site of vector at the 5' end of the promoter-AAS fragment = pGEM3ZAAS*Sac*(8)

c. Digested pGEM3ZAAS*Sac* with *Eco*RI and isolated 4.8kb promoter fragment and 1.4kb promoter-AAS coding region fragment.

d. Ligated 1.4kb fragment to pGEM3Z= pGEM3Z1.4kbAAS (1)

e. Designed downstream PCR primer located just 5' to the AAS coding sequence start, incorporating a *Sma*I site into the primer sequence- Called AASPROM1 (5'-TTTCCCGGGTATGGATACAAGCTG-3')

f. Used AASPROM1 primer with T7 promoter primer in a PCR using the pGEM3Z1.4kbAAS clone with the *Eco*RI fragment in the required orientation to produce a 300bp fragment. Expand proofreading polymerase mixture used in PCR

g. 300bp frag. representing the sequence from the *Eco*RI site in the AAS promoter immediately 5' to the AAS coding sequence start, digested with *Eco*RI and *Sma*I.

h. Ligated to *Eco*RI/*Sma*I digested pGEM3Z = pGEM3ZAASPCR fragment (4).

i. Digested pGEM3ZAASPCR fragment with *Eco*RI and *Sma*I to release 300bp AAS PCR fragment.

j. Ligated to *Eco*RI/*Sma*I digested pSCV1.6= pSCV1.6AASPCR fragment (1).

k. Digested pSCV1.6AASPCR fragment with *Eco*RI

l. Ligated to 4.8kb AAS EcoRI fragment and isolated recombinant carrying fragment in correct orientation to reconstruct 5.0 kb AAS promoter fragment = **pSCV1.6AASP** (6).

The gene regions including promoter sequences, obtained as described above, were then sequenced and the results are shown in Figures 3 and 5 respectively.

EXAMPLE 2: INTRODUCTION OF PROMOTERS INTO PLANTS

An efficient apple transformation system using disarmed strains *of Agrobacterium tumefaciens* carrying binary vectors (see James et al (1989) Plant Cell Reports 7:658-661; also James et al (1991) Plant Tissue Culture Manual B8:1-18, Kluwer Academic Publishers, Netherlands), was used to produce transgenic plants of the cultivar Greensleeves in which the *uidA* (or *gusA*) marker gene (encoding β-glucuronidase - GUS) is under the control of the ABG1 and AAS promoter fragments described here.

Transgenic fruit may be analyzed for GUS activity to assess promoter activity, for instance using methods analogous to those disclosed for measuring transgene expression in fruit tissue using constitutive promoters (James et al (1996) Bio/Technology 14:56-60).

Once the ripening-specific promoters driving a useful transgene have been introduced into a commercial apple cultivar apple, the transgenic clone with the desired properties may be clonally propagated using methods well known in the art.

EXAMPLE 3: APPLICATIONS FOR TRANSFORMANTS

In genetically improved transgenic apple plants, the storage qualities of the fruit may be improved by the expression of transgenes driven by the ripening-specific promoters. Using antisense or co-suppression strategies to down-regulate apple genes involved in ripening (e.g. genes involved in ethylene biosynthesis or cell wall degradation), the ripening process may be delayed, thus improving the storage life of the fruit. This strategy has successfully been applied to tomatoes to produce a marketable product. To combat post-harvest losses of fruit due to fungal rots, fruit-specific expression of fungal-resistance

transgenes (e.g. β-1,3-glucanases, chitinases) may be more effective than treatment with chemical fungicides because the anti-fungal molecules will be located in every cell rather than applied as a thin coating to the fruit skin. Therefore, even slightly damaged fruit will be less susceptible to rots. Such transformants will have advantages over existing systems. For instance certain traditional apple varieties have poor storage qualities (e.g. Queen Cox) which is a major commercial drawback. Genetic manipulation using the promoters described above provides a means to control the ripening process through targeted down-regulation of the genes involved. This concept, which is impossible using existing strategies, has previously been proved only in tomato and melon. Delayed fruit ripening caused by the expression of transgenes under the control of the fruit-specific ABG 1 and AAS promoters is likely to increase the storage life of fruit and boost profits for the industry.

Another post-harvest problem is storage rot which accounts for substantial losses to the industry. At present this phenomenon is controlled to some degree by the application of chemical fungicides. As well as being expensive, these treatments are becoming less acceptable to consumers who are demanding a reduction in the use of chemicals on food. Targeted expression of non-toxic fungal-resistance factors using the fruit-specific ABG 1 and AAS promoters could reduce post-harvest fruit losses and should break the reliance on chemicals to control storage rots.

CLAIMS

1. A recombinant polynucleotide comprising a promoter sequence being: (a) an inducible promoter obtainable from apple, or (b) a functional portion therof, or (c) a functional derivative or homolog promoter being at least 70% homologous to either.

2. A recombinant polynucleotide according to claim 1 which comprises the sequence of Figure 3 or Figure 5, or (b) a functional portion therof, or (c) a functional derivative or homolog promoter being at least 70% homologous to either.

3. A polynucleotide as claimed in claim 1 or claim 2 wherein the promoter sequence is activated in response to tissue specific agents.

4. A polynucleotide as claimed in claim 3 wherein the agents are specific to fruit.

5. A polynucleotide as claimed in claim 4 wherein the agents are specific to ripening fruit.

6. A polynucleotide as claimed in any one of the preceding claims wherein the inducible promoter is the Apple β-Galactosidase (ABG1) promoter, or the 1-AminoCyclopropane-1-Carboxylate synthase (ACC Synthase) promoter.

7. A polynucleotide as claimed in claim 6 wherein the inducible promoter is the ABG1 promoter where the full length ABG1 gene includes an EcoRI fragment of SEQ ID NO 10 as shown in Figure 1(1) and a PstI fragment of SEQ ID NO 11 as shown in Figure 1(2).

8. A polynucleotide as claimed in claim 6 wherein the inducible promoter is the ACC synthase promoter wherein the ACC synthase gene comprises SEQ ID NO 13 as shown in Figure 2.

9. An isolated polynucleotide which comprises the ABG1 promoter or ACC promoter or a functional portion thereof.

10. An isolated polynucleotide according to claim 7 which comprises a portion of SEQ ID NO 1 or SEQ ID NO 2 which can act as an inducible promoter.

11. An isolated polynucleotide according to claim 8 which comprises a portion of SEQ ID NO 1 as shown in Figure 3 which includes SEQ ID NO 4 as shown in Figure 8.

12. An isolated polynucleotide according to claim 8 which comprises a portion of SEQ ID NO 2 as shown in Figure 5 which includes SEQ ID NO 3 as shown in Figure 8.

13. An isolated polynucleotide which comprises the sequence GAAAAATCACATTTTTTACACTAAAAAG (SEQ ID NO 7) or a derivative thereof.

14. A replication vector comprising a polynucleotide as claimed in any one of the preceding claims further comprising a replication element which permits replication of the vector in a host cell.

15. An expression vector comprising a promoter sequence which comprises a polynucleotide as claimed in any one of claims 1 to 13.

16. A vector as claimed in claim 15 further comprising a heterologous gene operatively linked to said promoter sequence.

17. A vector as claimed in claim 16 wherein said heterologous gene encodes any of: (a) antisense RNA capable of down-regulating genes involved in ripening; (b) a peptide or protein improving fungal, insect, bacterial, viral, herbicidal, nematode, or

arachnid resistance; (c) a detectable or selectable marker protein.

18. A vector as claimed in any one of claims 7 to 13 comprising elements derived from the Ti plasmid.

19. A host cell containing a vector as claimed in any one of claims 14 to 18.

20. A host plant cell transformed with a vector as claimed in any one of claims 14 to 18.

21. A method of producing a transgenic plant comprising regenerating a plant from the transformed host cell of claim 20.

22. A transgenic plant comprising a host cell as claimed in claim 20.

23. A transgenic apple plant as claimed in claim 22 or produced by the method of claim 21.

24. A method of producing apples having a modified phenotype, said method comprising cultivating the transgenic apple plant of claim 23 and harvesting the fruit of the plant.

25. An apple produced by the method of claim 24.

26. A probe comprising SEQ ID NO 3 or SEQ ID NO 4 as shown in Figure 8 or a part thereof or a derivative which hybridises to said sequence under stringent conditions.

27. A method of separating a transcription factors from fruit cells which method comprises immobilising a probe according to claim 26 and exposing said immobilised probe to a sample containing extracted nuclear proteins from fruit cells.

28. A method of isolating a ripening specific promoter sequences from plant DNA, said method comprising probing a plant DNA library with a probe according to claim 26.

29. A ripening specific promoter sequence obtained by a method according to claim 28.

30. A probe comprising SEQ ID No 5 as shown in Figure 8 or a portion or derivative thereof which hybridises to said sequence under stringent conditions.

Fig.1.

1. ABG *Eco*RI fragment Forward

```
----------------------------------------------------------------------------
GATTCTTATTCCACATGTATTACACAAACATAAATTAGGAAGTTCTTCCTCTCCTAGGAATCCAAATCCTCAAAGGTTTT

----------------------------------------------------------------------------
CCCTCTCTAAGGAATCCTATTCCTCATAGCCTTGCGACGTTTATATATACGGATTCACCATACAAATGAAATACAACAGA

----------------------------------------------------------------------------
TACAGTATTTTCTACAGACACCCGTATTTCCAAAAATGTGAGAGAGCTTGTTCAAGGCAGCAGAAGTGGCCCCCCCCCT

----------------------------------------------------------------------------
TCCCTTTGCAGTTTGCACATTTGCAATCTTCATCTTCAAAACCTTATTTAAAGCAGTAGCGGGAGCAACACGTTCCTTGC

                                                    start of cDNA sequence
----------------------------------------------------------CTCAACTCTGCCACTCTCTCTCTG
ACAACCATTGAACCAAACCATAAACTTTCTCACCCGTGAAATCCAGCAGTACACTTCTCTACTCTGCCACCCCCTTNCTG

TCTCTTTCCAAAATATCAAAAGCACCAAACAAAAGAAA----CCAAATTC-AAATCCCAAAACACAA-TATATATT--TT
TCTCTTTTCCGAATACCAAANGCACCAACCAAAGAAACANTTCCAANTCCCAAACCCAAAAAAATAAATATGTATTATTC

AAGTTTTTGGTACAAACAAAG-CAAGT-ATATTTATATATAAAGGCCATTGCTTTTGAGCGTTTCAGAAGCAAGGGAAAA
AAGTTTTGGGTACAAACAAANGCAANTTATNTNTATTATTNANGGCCATTGCTTTNGGGATTTTCAGAAGCAGGGGACAA

 1
 M
ATG   SEQ ID NO.8
A--   SEQ ID NO.10
```

Fig.1 (Cont).

2. ABG PstI fragment Forward

```
   21
   A  A  S  A  S  V  S  Y  D  H  K  A  I  I  I  N  G  Q  K  R  I  L  I  S  G  S
CTGCAGCTTCAGCTTCTGTGAGTTATGACCACAAGGCTATAATAATTAATGGGCAGAAAAGGATTTTAATTTCTGGCTCC
CTGCAGCTTCAGCTTCCGTGGGTTATGACCACAAAGCTATAATAATTAATGGGCAGAGAAGGATTTTAATTTCTGGATCA
                  G                                                R
                                55
 I  H  Y  P  R  S  T  P  E
ATTCACTATCCCAGAAGCACTCCCGAG----------------------------------------------------
ATTCACTATCCCAGAAGCACTCCTGAGGTACTTTATACAATGCCAATGTGGTTCTTCTTTGTTTCAATATTTTCTGGGTT

----------------------------------------------------------------------------------
TTTTTTATCTTTTTTTTCTGGTGCAATTTTTTACATCTGGGTTTTTTTTCAGTTCAAATTTCTGACACTGGGGGATATAT

----------------------------------------------------------------------------------
TATATATTATTTATTATTTGGATTTTTAACTTGTGGGAGACTATGATCTTGAATTAAAAAGTTTCAGAATGTTTTNTCGG

                                                    56
                                                    M  W  P  D  L  I  Q  K  A  K
--------------------------------------------------ATGTGGCCGGATTTAATTCAGAAGGCCAAAG
CTCAAATTATTGATATTTNGGGTTTAATTTTTGGTGTGTGTGGTTGCAGATGTGGNCGGATTTGATTCAGAAGGCCAAAG

                                                              86
D  G  G  L  D  V  I  Q  T  Y  V  E  W  N  G  H  E  P  S  P  G
ATGGAGGCTTGGATGTTATACAGACCTATGTGTTTTGGAATGGCCATGAACCTTCTCCGGGA----------------
CTGGAGGNTTGGNTGTTATACAGACCTATGTGTTTNGGNATGGNCATGAACCTTCTCCAGGAAAAGTAATNNAATTAACA
A

---------------------------------------------------- SEQ ID NO.9
ANNTGGTTCTGAATTNNAGATTTNCTNATCTCTAATGNAAATNTTNATGNTAANNAN SEQ ID NO.11
```

Fig.2.

1. *XhoI* deletion-Reverse

```
----------------------------------------------------------------------
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----------------------------------------------------------------------
TAGGAAAGGGAACCAGGAATTTTAANGAGATTTTTTAAAAAGGAGATTNTTCATAATNNNTTTNTTNAGG

----------------------------------------------------------------------
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----------------------------------------------------------------------
AGAAGGCAACAATTTTAAGATAATNTCCTNAACATTTATAAAAAATATGAANANTCAGTGGGANGTGTCA

----------------------------------------------------------------------
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                                        start of cDNA sequence
----------------------------------------ATCCAAAACCAAAACCTCAAACTCTCTC
CCTCCAGTCCAACAACATCCAATATCCCANTTCAAACTTGTAATCCAAAACCAAAACCTCAAACTNTNTN

TCTATTGCTTTCTCTTCCTTTCCACACTTCTTTCTTACAGCTTGTATCCATACACAAGAAAATTAACCAA
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  1
```

Fig.2 (Cont).

M R M L S R N A T F N S H G Q D S S Y F L G W
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AATGCGCATGTTATCCAGAAACGNTACGTTCAANTNTCACGGCCAAGACTCCTCCTACTTCTTAGGTTGG

Q E Y E K N P Y H E V H N T N G I I Q M G L A
CAAGAGTATGAGAAGAACCCCTACCATGAGGTCCACAACACAAACGGGATTATTCAGATGGGTCTAGCAG
CAAGAGTATGAGAAGAACCCCTACCATGAGGTCCACAACACAAACGGGATTATTCAGATGGGTNTAGCAG
49

E N Q
AAAATCAG----------
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50

L C F D
----------CTCTGTTTTGAT
TATGTAGTTATATTTAGTATATAAACTTTGTGCGCGTTTCAATATTTTTTTTTTCTAGCTCTGTTTTG-T

L L
CTTCTC SEQ ID NO.12
CTTCTC SEQ ID NO.13

Fig.3.

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CAAGACATGTCCACAACTTTGAGCCGGAAGGACTTACGCATGACACCTAACTGTCAGCT
ACCATCAGTATGGATTTACGTAGGAATTTACATATCACATAAAAAATGACTACTGACAACA
GGACTCAAACCTTGGGGGGACGGCACACAAAGTAAAGATCTTACCTACTAAGCCAACC
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CATAACGATAATAGTTTGAACCTTGATGGTTAAATGGAGAAAAAATGAGAAGAAAACAAA
ATTTCCATGCAACATCGATTGCACACGTGTCGGACCACGATCGGTAGCTAGCTTCAATG
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SEQ ID NO.1

Fig.4(i).

ABGIP β-galactosidase

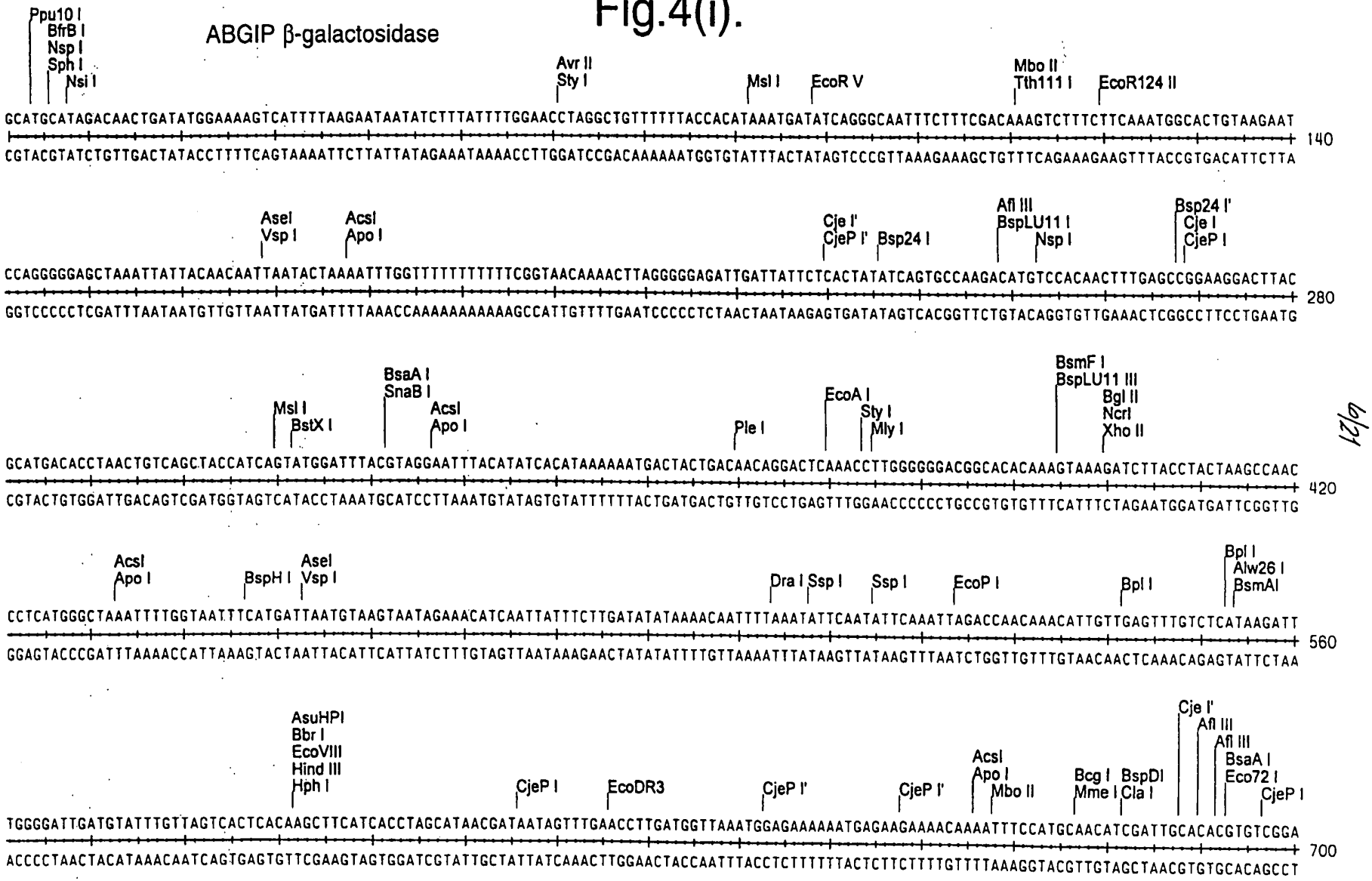
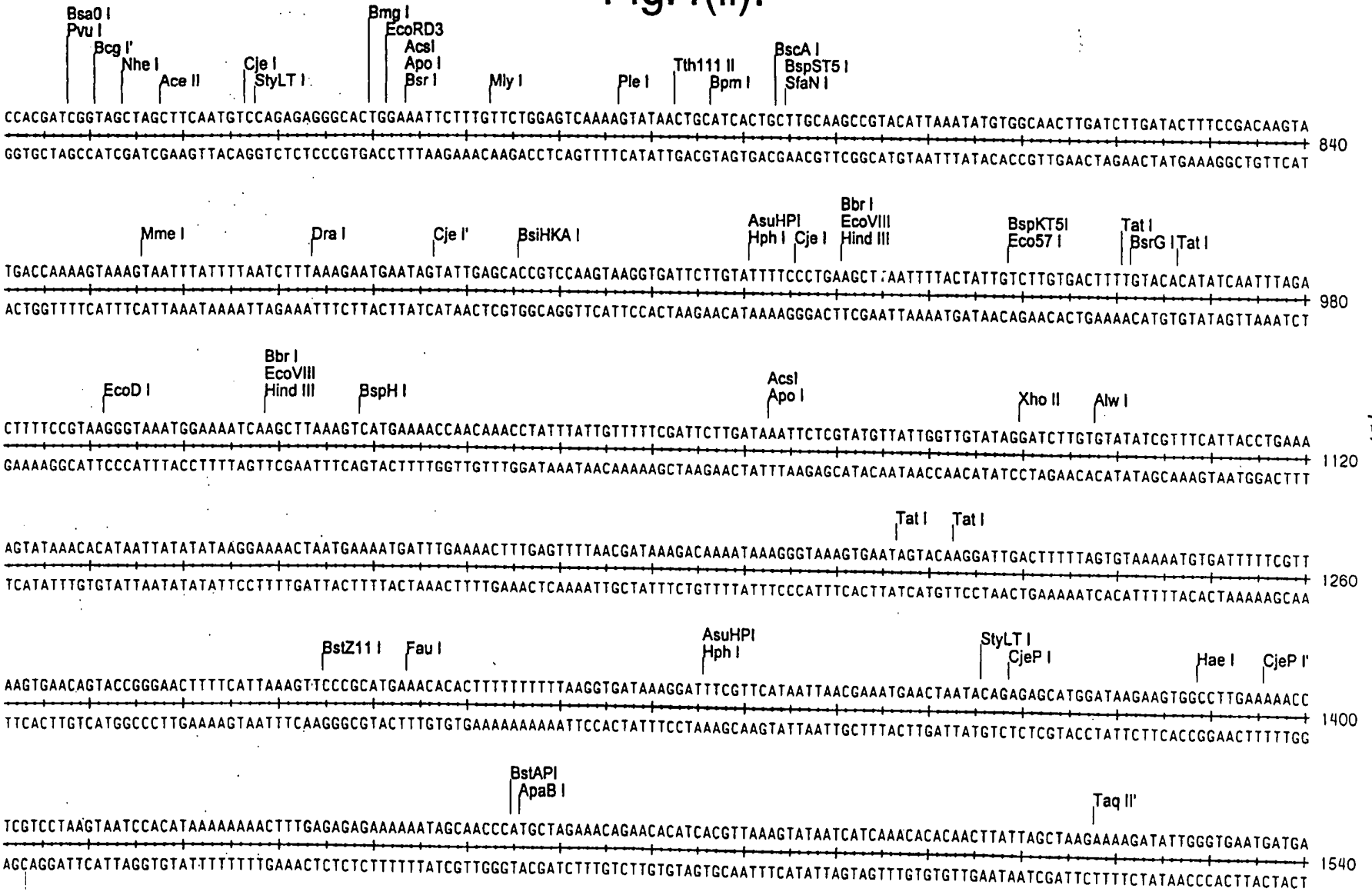


Fig.4(ii).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Bcg I'
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Cje I
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Mly I
Ple I
Tth111 II
Bpm I
BscA I
BspST5 I
SfaN I
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Dra I
Cje I'
BsiHKA I
AsuHPI
Hph I
Cje I
Bbr I
EcoVIII
Hind III
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Eco57 I
Tat I
BsrG I
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Bbr I
EcoVIII
Hind III
BspH I
AcsI
Apo I
Xho II
Alw I
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Fau I
AsuHPI
Hph I
StyLT I
CjeP I
Hae I
CjeP I'
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1400
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Taq II'
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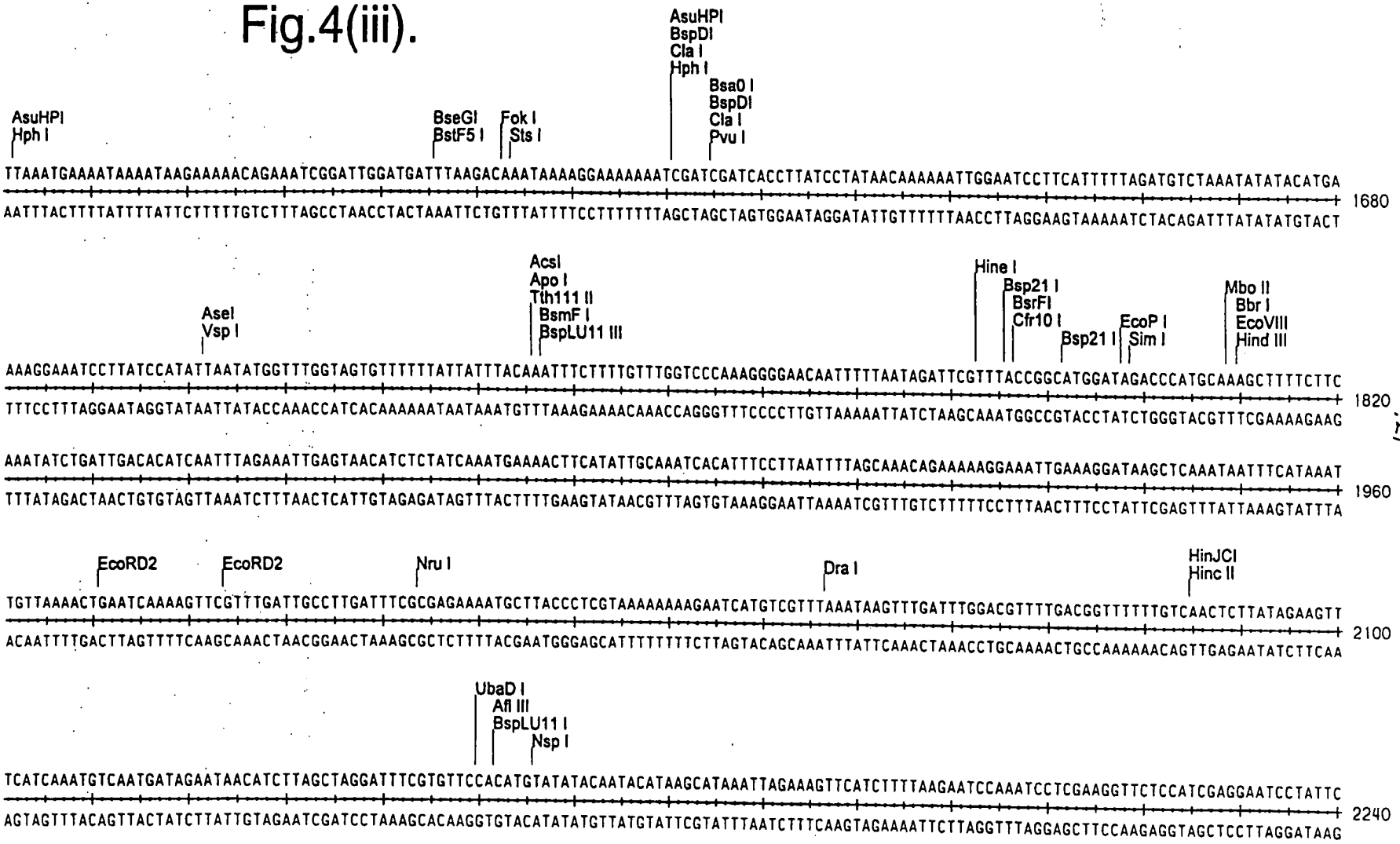
Fig.4(iii).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Vsp I
AcsI
Apo I
Tth111 II
BsmF I
BspLU11 III
Hine I
Bsp21 I
BsrFI
Cfr10 I
Bsp21 I
EcoP I
Sim I
Mbo II
Bbr I
EcoVIII
Hind III
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1960
EcoRD2
EcoRD2
Nru I
Dra I
HinJCI
Hinc II
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Afl III
BspLU11 I
Nsp I
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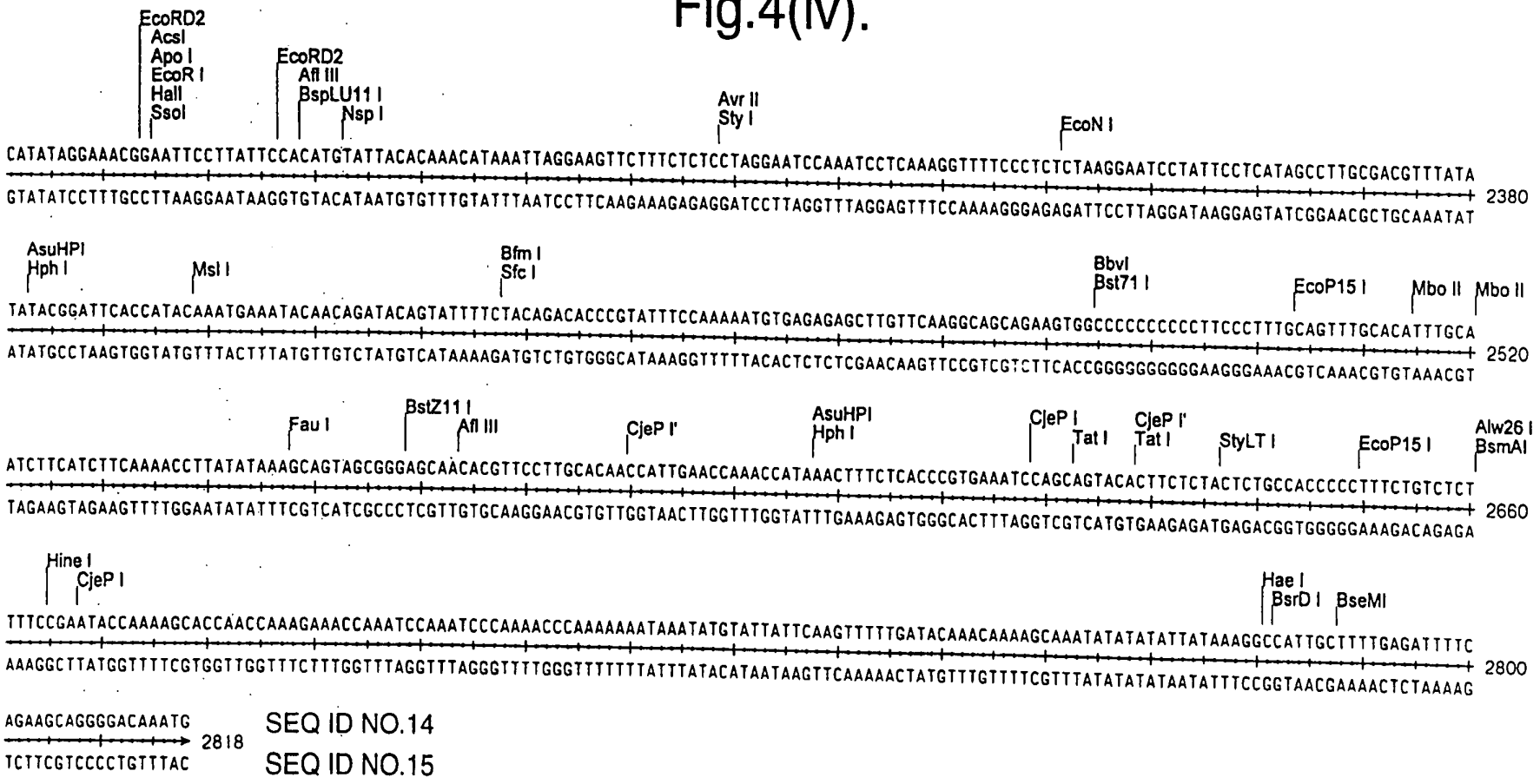
Fig.4(iv).



SEQ ID NO.14

SEQ ID NO.15

Fig.5.

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Fig.5 (Cont).

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Fig.6(i).

AASP ACC Synthase

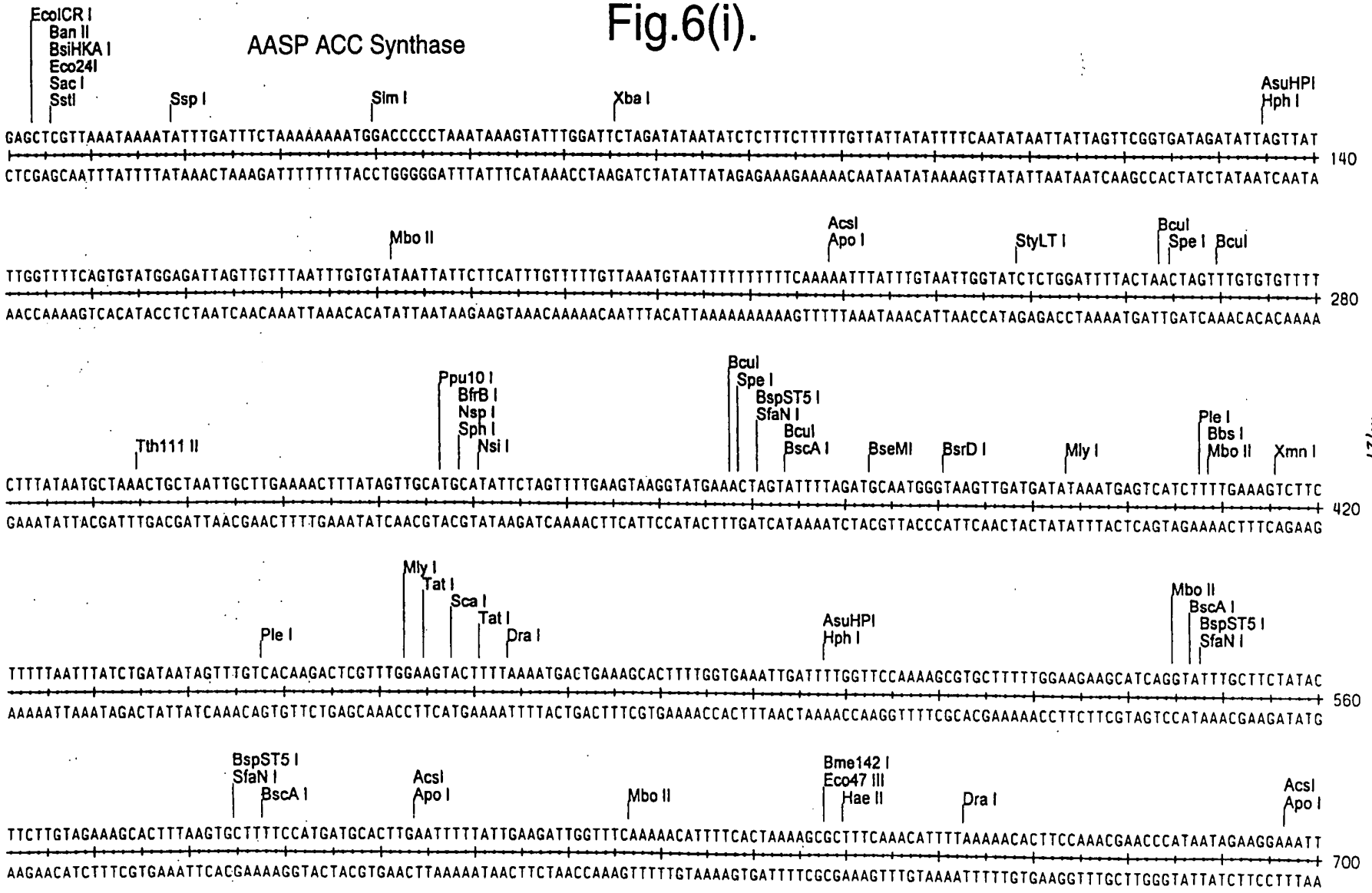

Fig.6(ii).

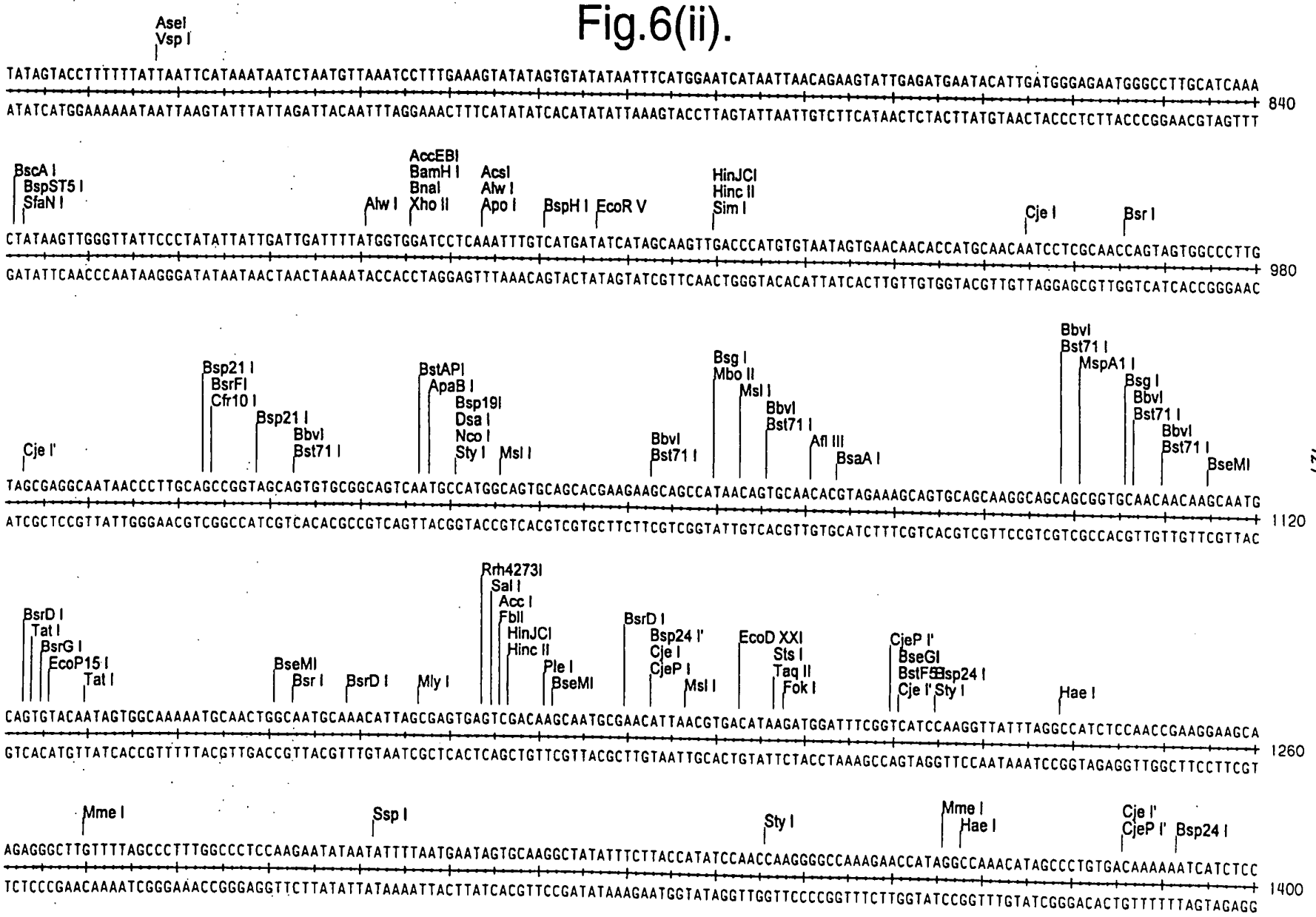
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Fig.6(iii).

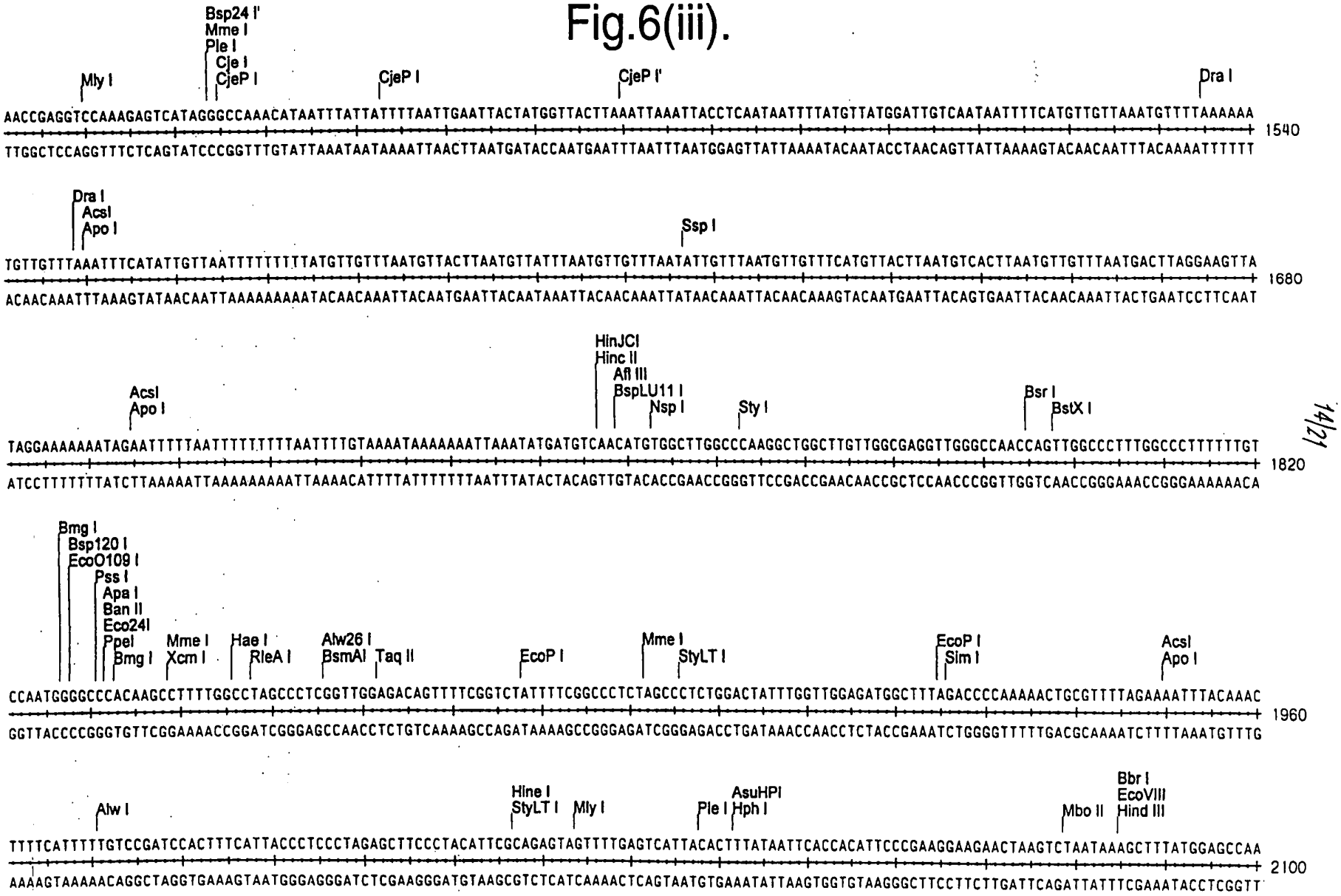
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Fig.6(iv).

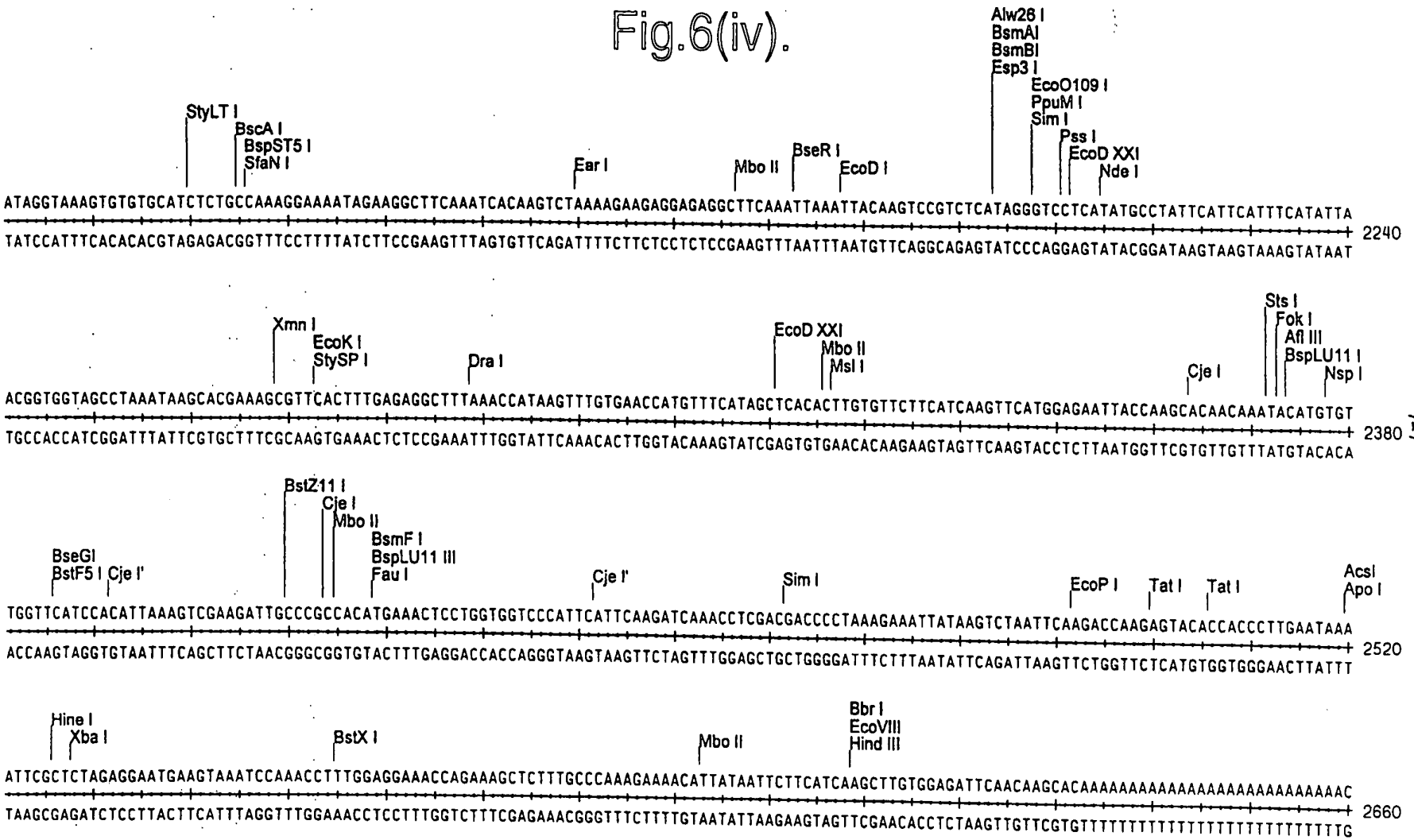
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Fig.6(v).

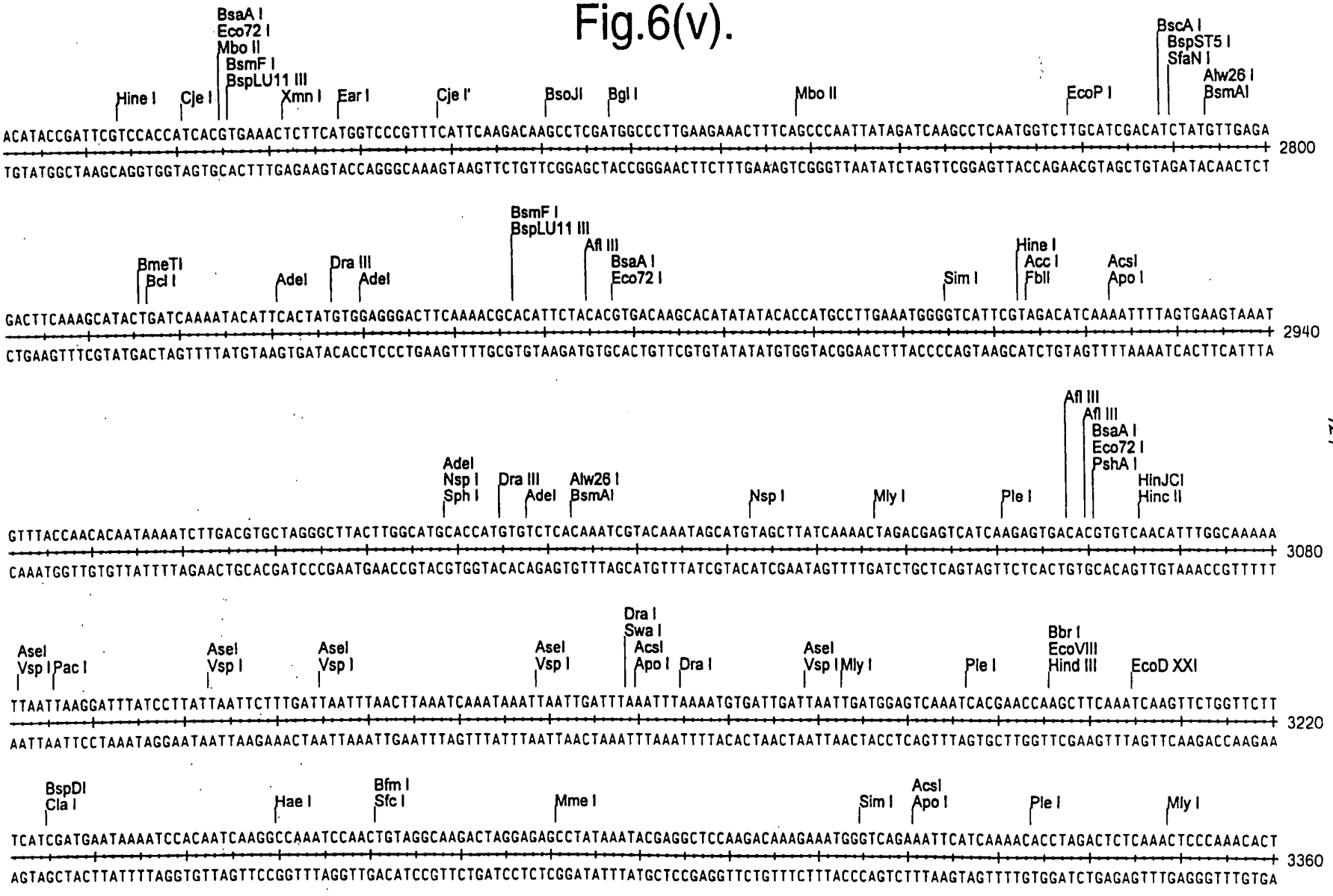

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Fig.6(vi).

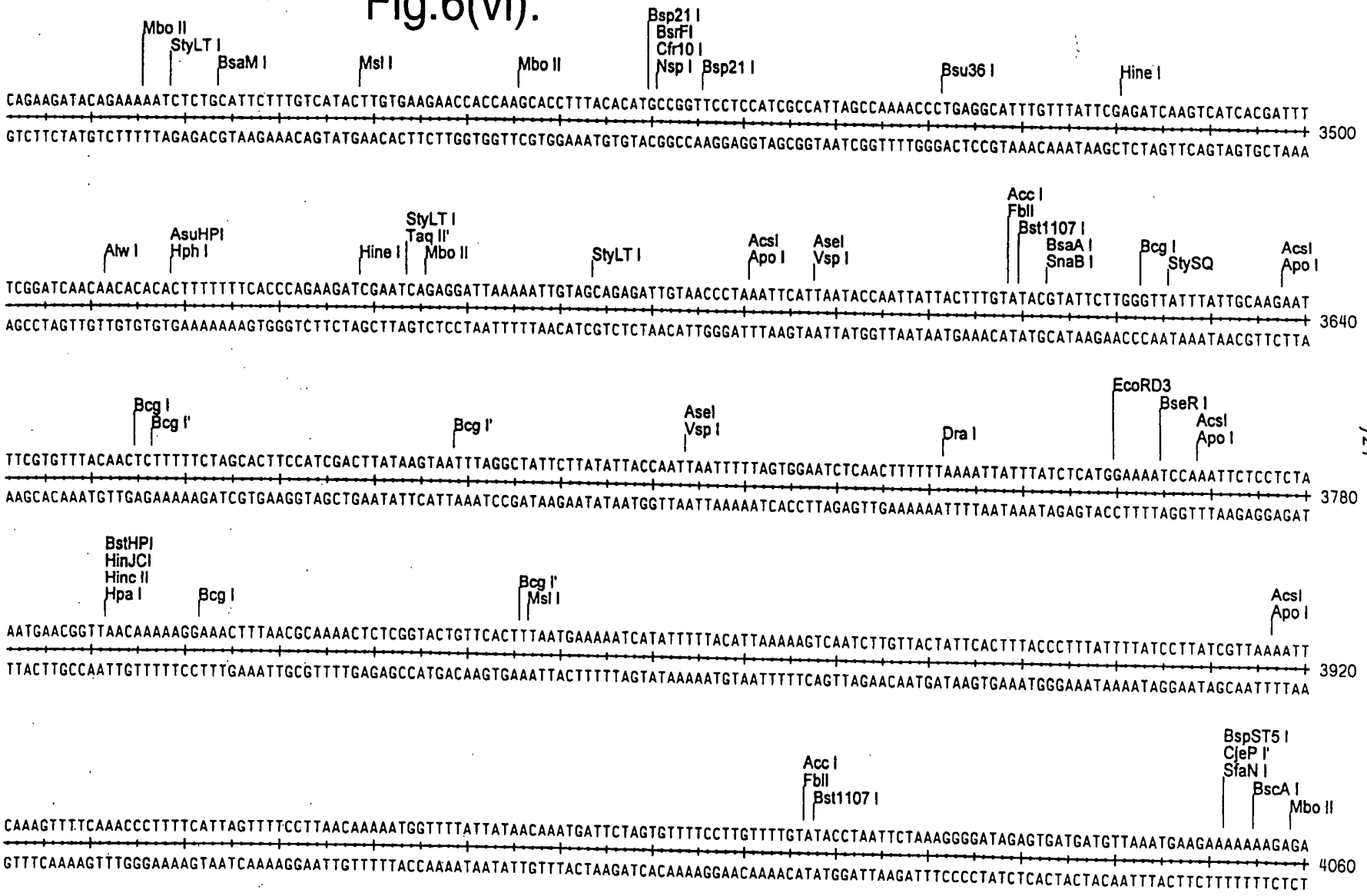
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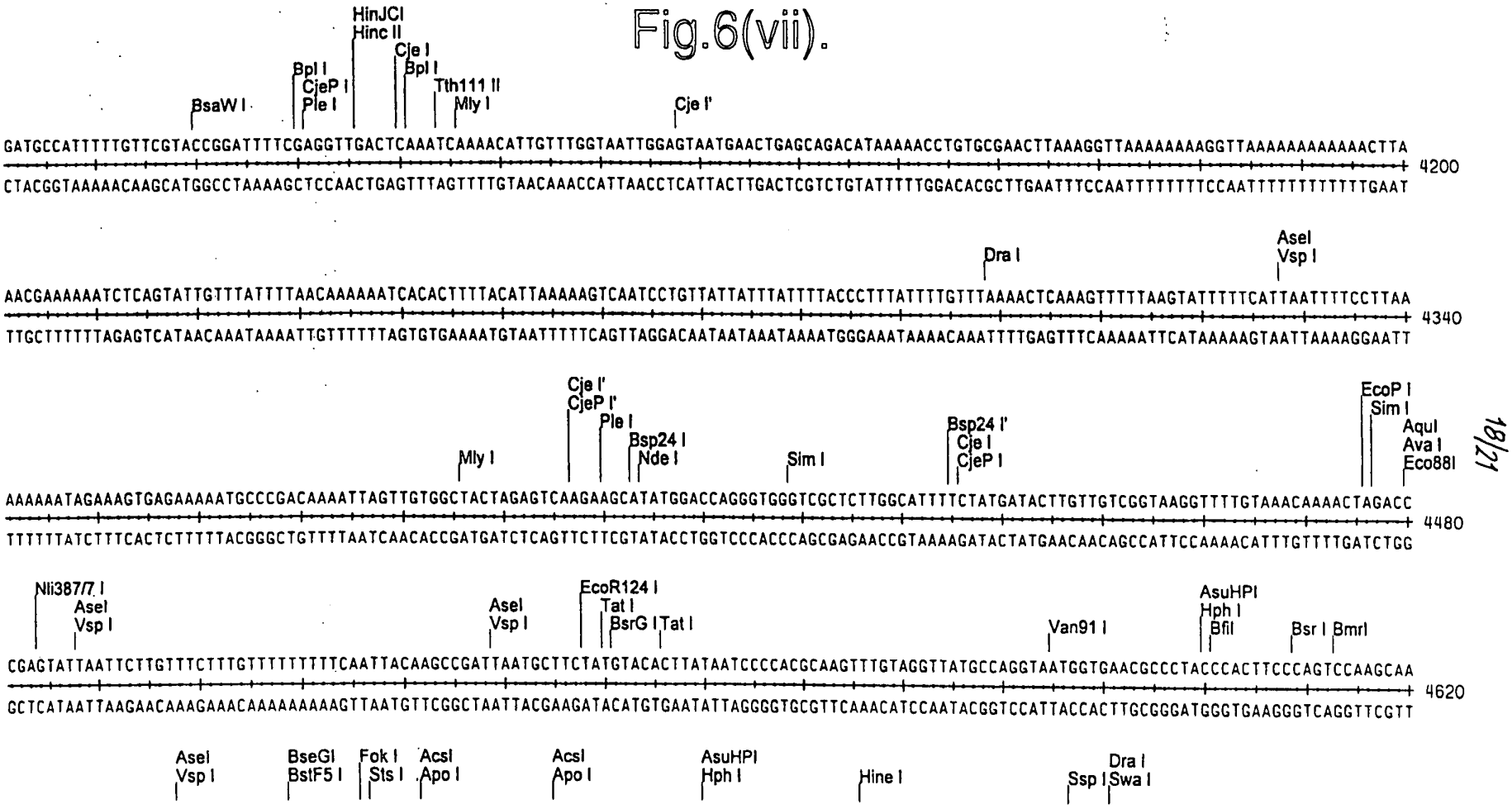
Fig.6(vii).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Eco88I
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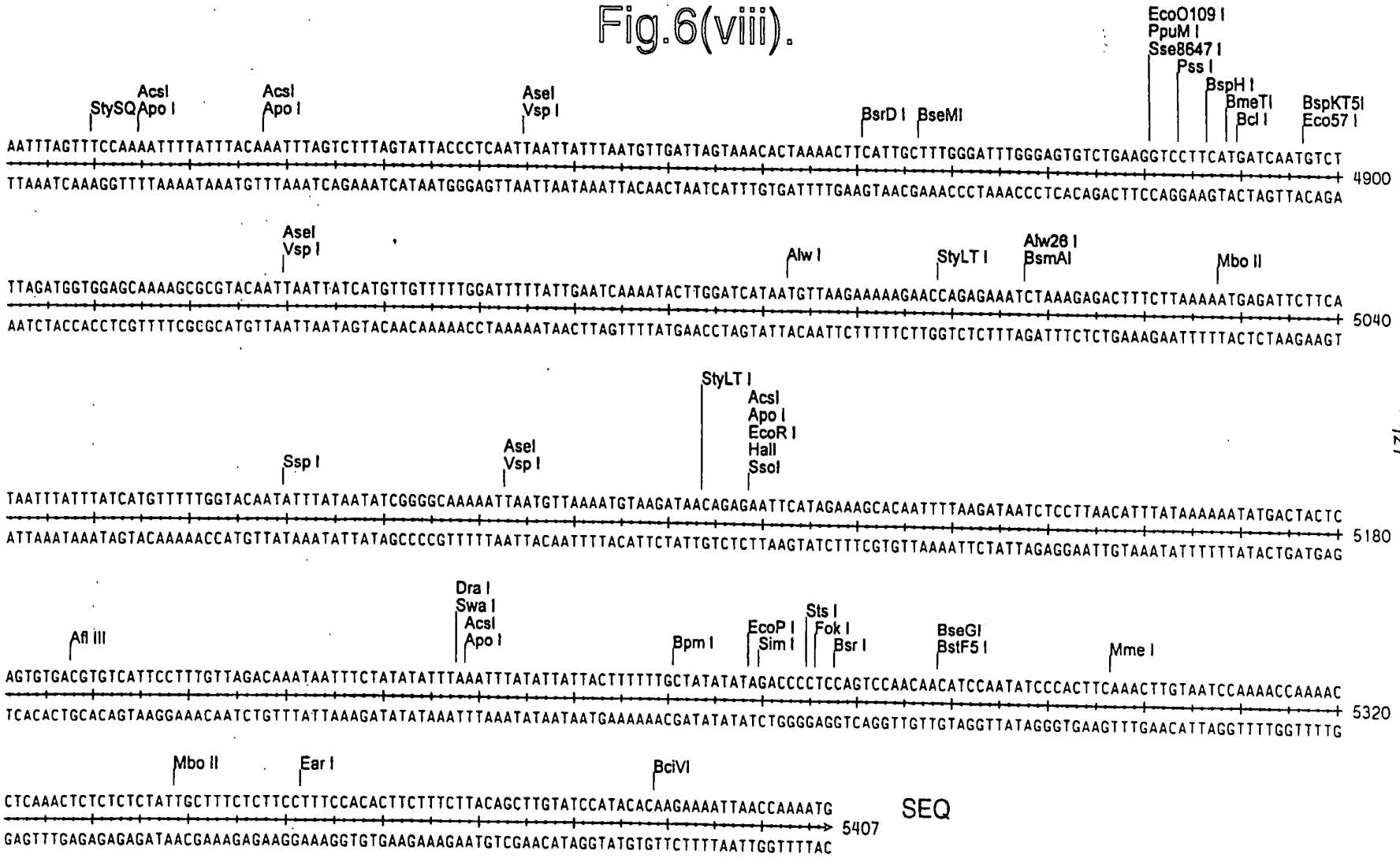
TTTTTTATCTTTCACTCTTTTTACGGGCTGTTTTAATCAACACCGATGATCTCAGTTCTTCGTATACCTGGTCCCACCCAGCGAGAACCGTAAAAGATACTATGAACAACAGCCATTCCAAAACATTTGTTTTGATCTGG
4480
Nli3877 I
AseI
Vsp I
AseI
Vsp I
EcoR124 I
Tat I
BsrG I
Tat I
Van91 I
AsuHPI
Hph I
BfiI
Bsr I
BmrI
CGAGTATTAATTCTTGTTTCTTTGTTTTTTTTTCAATTACAAGCCGATTAATGCTTCTATGTACACTTATAATCCCCACGCAAGTTTGTAGGTTATGCCAGGTAATGGTGAACGCCCTACCCACTTCCCAGTCCAAGCAA
GCTCATAATTAAGAACAAAGAAACAAAAAAAAAGTTAATGTTCGGCTAATTACGAAGATACATGTGAATATTAGGGGTGCGTTCAAACATCCAATACGGTCCATTACCACTTGCGGGATGGGTGAAGGGTCAGGTTCGTT
4620
AseI
Vsp I
BseGI
BstF5 I
Fok I
Sts I
AcsI
Apo I
AcsI
Apo I
AsuHPI
Hph I
Hine I
Dra I
Ssp I
Swa I
ATAGTGAGAAAATAAATTAATGGATGATACTAGGAAAATTAAATTTGGAGATAAAATTTGCAAATTATATAATATGTCACCTATACGAATTAACACATTTATCAATATTTAAATAATAAATCAATCATCAACTACCATAT
TATCACTCTTTTATTTAATTACCTACTATGATCCTTTTAATTTAAACCTCTATTTTAAACGTTTAATATATTATACAGTGGATATGCTTAATTGTGTAAATAGTTATAAATTTATTATTTAGTTAGTAGTTGATGGTATA
4760

Fig.6(viii).



SEQ

Fig.7.

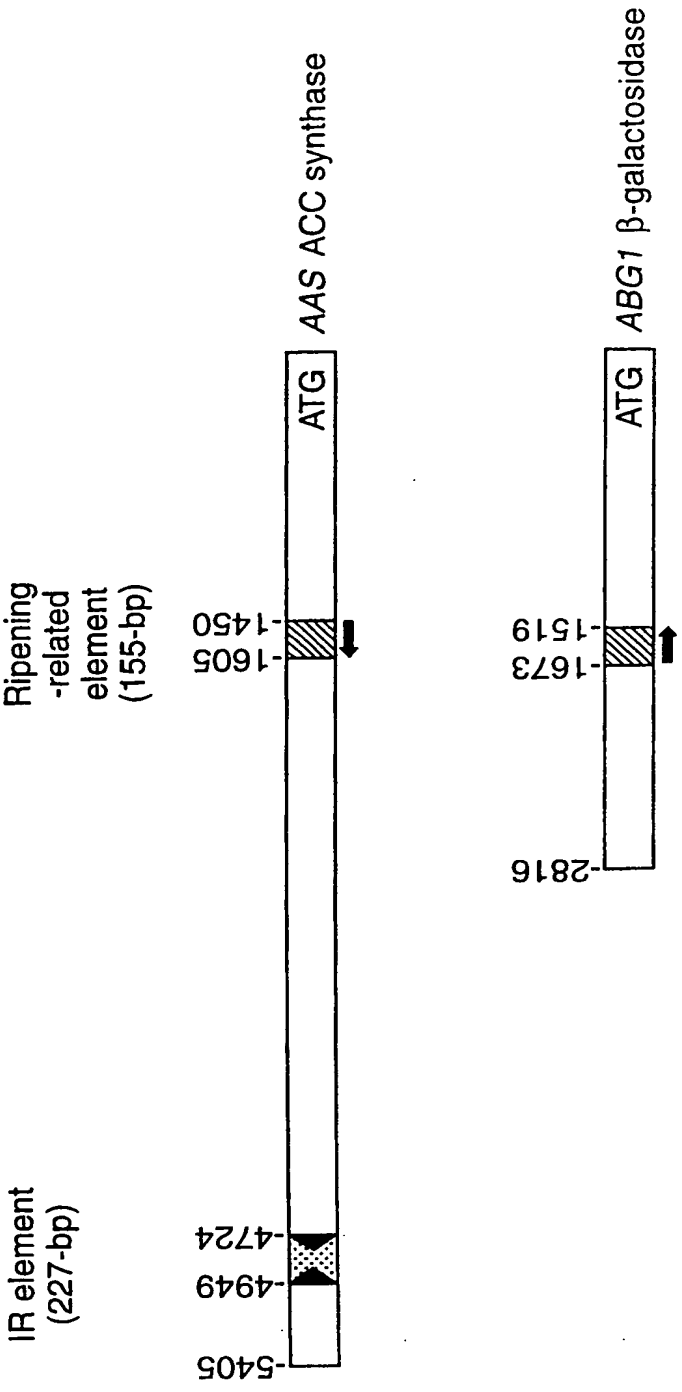
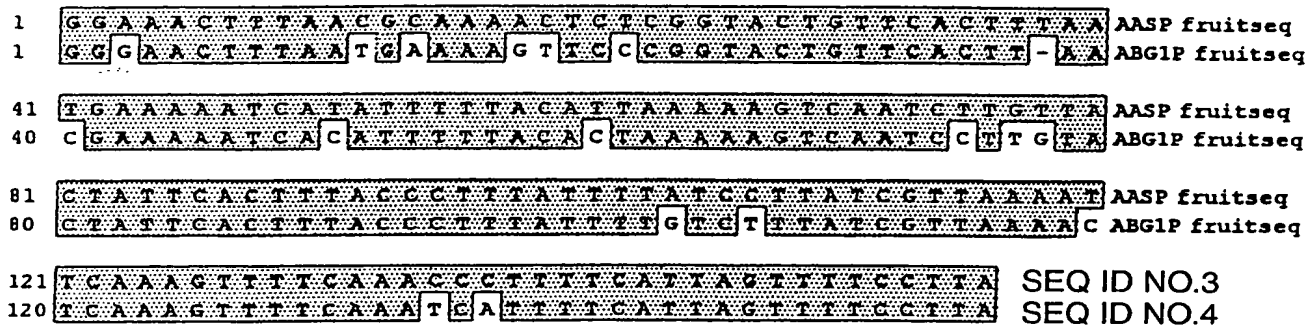
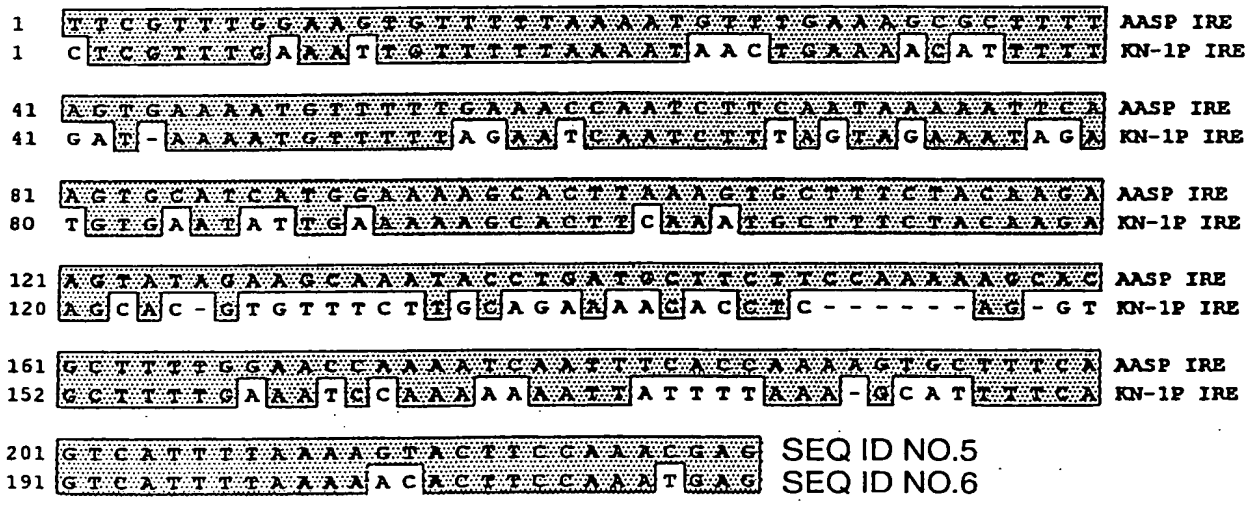

A
Ripening
-related
element
(155-bp)
B
IR element
(227-bp)
AAS ACC synthase
ATG
-1450
-1605
-4724
-4949
-5405
ABG1 β-galactosidase
ATG
-1519
-1673
-2816

Fig.8.

A: Alignment of ripening-related elements found in the *ABG1* and *AAS* promoters



B: Alignment of the inverted repeat (IR) element of the *AAS* promoter with that found in the apple Kn-1 knotted gene homologue promoter



INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/GB 98/01000

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C12N15/55 C12N15/11 C12N15/82 C12N5/10
C12Q1/68 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEN, HOUQI ET AL: "Nucleotide sequence of an apple gene encoding a light-harvesting chlorophyll a/b binding polypeptide of photosystem II." NUCLEIC ACIDS RES., (1990) VOL. 18, NO. 3, P. 679., XP002070929 see the whole document ---	1,14-16, 18-22
Y	ROSS G S ET AL: "Apple beta-galactosidase. Activity against cell wall polysaccharides and characterization of a related cDNA clone." PLANT PHYSIOLOGY, (1994 OCT) 106 (2) 521-8. JOURNAL CODE: P98. ISSN: 0032-0889., XP002070930 cited in the application see the whole document --- -/--	1-6,9, 14-25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
9 July 1998	21/07/1998

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Ir .ational Application No
PCT/GB 98/01000

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A	WO 92 04456 A (US OF AMARICA REPRESENTED BY T) 19 March 1992 * see the whole document, esp. examples 1, 3-5 *	1-30
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In ational Application No
PCT/GB 98/01000

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Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

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